

Amendments to the Specification:

Please amend the specification by inserting the substitute Sequence Listing, as attached hereto, into the above-identified patent application.

Please cancel the paragraph bridging pages 4 and 5 and, in its place, add the following new paragraph:

Although PKC could be assayed perfectly well using a peptide comprising the said epitope (Table 1), this substrate was inferior to other substrates that have been used to assay this protein kinase. This is considered to be because PKC prefers basic residues C-terminal to the site of phosphorylation, which are not present in the epitope, in addition to basic residues N-terminal to the site of phosphorylation [5]. A preferred epitope for isoforms of PKC and related protein kinases, such as AGC family members may comprise or consist of the seven amino acids starting with the serine of the peptide substrate RRRLSFAEPG (SEQ ID NO:4).

Please cancel the paragraph at page 7, lines 14-28, and, in its place, add the following new paragraph:

- 4 -

It is preferred that the consensus sequence is Arg-Arg-Arg-Xaa-Ser (SEQ ID NO:1), Arg (or Lys)-Xaa-Arg-Xaa-Xaa-Ser (SEQ ID NO:2), Hyd-Xaa-Arg-Xaa-Xaa-Ser (SEQ ID NO:3) or Xaa-pSer-Xaa-Xaa-Ser (SEQ ID NO:5). In each case the consensus sequence is positioned so that the C-terminal serine of the consensus sequence (which is the serine which is phosphorylated by the protein kinase recognising the consensus sequence) is the serine of the phosphorylatable portion. In a further preferred embodiment the phosphorylatable portion has the amino acid sequence LSFAEPG (SEQ ID NO:6) sequence (which includes sequences with no, one, two, three, four or five residues (other than the serine) conservatively substituted). Thus, the leucine residue immediately N-terminal of the phosphorylatable serine residue corresponds to the "Xaa" residue immediately N-terminal of the serine residue in each of the consensus sequences indicated above. In particularly preferred embodiments, the protein kinase substrate polypeptides are polypeptides of the invention, as discussed further below.

Please cancel the paragraph bridging pages 11 and 12 and, in its place, add the following new paragraph:

- 5 -

A further aspect of the invention provides an antibody specific for the epitope formed by the amino acid sequence LSFAEPG (ie with the serine unphosphorylated) (SEQ ID NO:6). A further aspect of the invention provides an antibody specific for the epitope formed by the amino acid sequence LpSFAEPG (ie with the serine phosphorylated) (SEQ ID NO:7).

Please cancel the paragraph at page 12, lines 4-6, and, in its place, add the following new paragraph:

By "specific" is included the meaning that the antibody binds to the epitope formed by the amino acid sequence LSFAEPG (SEQ ID NO:6) but not to the epitope formed by the amino acid sequence LpSFAEPG (SEQ ID NO:7) or vice versa.

Please cancel the paragraph at page 12, lines 12-26, and, in its place, add the following new paragraph:

A further aspect of the invention provides a polypeptide of less than 40, 30, 20, 19, 18, 17, 16, 15, or 14 amino acids in length wherein the polypeptide is not a fragment of glycogen synthase kinase 3, and wherein the polypeptide comprises the amino acid sequence LSFAEPG (SEQ

- 6 -

ID NO:6) (which includes sequences with no, one, two, three, four or five residues (other than the serine) conservatively substituted) and further comprising a specificity conferring portion comprising an amino acid sequence (which may overlap with the sequence LSFAEPG (SEQ ID NO:6)) corresponding to a consensus sequence for a protein kinase, wherein the sequence corresponding to the consensus sequence is positioned relative to the sequence LSFAEPG (SEQ ID NO:6) such that the protein kinase is capable of phosphorylating the polypeptide at the serine residue of the sequence LSFAEPG (SEQ ID NO:6). It is particularly preferred that the substrate polypeptide is 13, 12, 11, 10 or 9 amino acids in length. It is preferred that the amino acid sequence corresponding to the consensus sequence extends to the N-terminus of the sequence LSFAEPG (SEQ ID NO:6).

Please cancel the paragraph bridging pages 12 and 13, and, in its place, add the following new paragraph:

In particularly preferred embodiments, the consensus sequence is Arg/Lys-Arg/Lys-Arg/Lys-Xaa-Ser (SEQ ID NO:8), Arg/Lys-Xaa-Arg/Lys-Xaa-Xaa-Ser (SEQ ID NO:9), Hyd-Xaa-Arg-Xaa-Xaa-Ser (SEQ ID NO:3) or Xaa-pSer-Xaa-Xaa-Ser (SEQ ID

- 7 -

NO:5). In each case the consensus sequence is positioned so that the C-terminal serine of the consensus sequence (which is the serine which is phosphorylated by the protein kinase recognising the consensus sequence) is the serine of the LSFAEPG sequence (SEQ ID NO:6).

Please cancel the paragraph at page 13, lines 6-7, and, in its place, add the following new paragraph:

Arginines other than the Arg in Hyd-Xaa-Arg-Xaa-Xaa-Ser (SEQ ID NO:3) may be replaced with Lysine.

Please cancel the paragraph at page 13, lines 9-12, and, in its place, add the following new paragraph:

Thus, it is preferred that the polypeptide has or comprises the amino acid sequence Arg-Arg-Arg-Leu-Ser-Phe-Ala-Glu-Pro-Gly (SEQ ID NO:4), Arg-Xaa-Arg-Xaa-Leu-Ser-Phe-Ala-Glu-Pro-Gly (SEQ ID NO:29), Hyd-Xaa-Arg-Xaa-Leu-Ser-Phe-Ala-Glu-Pro-Gly (SEQ ID NO:30) or Xaa-pSer-Xaa-Leu-Ser-Phe-Ala-Glu-Pro-Gly (SEQ ID NO:12).

Please cancel the paragraph at page 13, lines 14-17, and, in its place, add the following new paragraph:

- 8 -

In particularly preferred embodiments, the polypeptide has the amino acid sequence Arg-Arg-Arg-Leu-Ser-Phe-Ala-Glu-Pro-Gly (SEQ ID NO:4), Arg-Ala-Arg-Thr-Leu-Ser-Phe-Ala-Glu-Pro-Gly (SEQ ID NO:16) or Lys-Lys-Leu-Asn-Arg-Thr-Leu-Ser-Phe-Ala-Glu-Pro-Gly (SEQ ID NO:13).

Please cancel the paragraph at page 13, lines 19-21, and, in its place, add the following new paragraph:

A further aspect of the invention provides a polypeptide of the preceding aspect of the invention in which the serine in the sequence LSFAEPG (SEQ ID NO:6) is replaced by phosphoserine (SEQ ID NO:7).

Please cancel the paragraph at page 18, lines 6-9, and, in its place, add the following new paragraph:

Thus, it will be appreciated that the substrate polypeptide, for example which comprises the amino acid sequence LSFAEPG (SEQ ID NO:6) may be a peptidomimetic compound, as described above, though this is not preferred.

Please cancel the paragraph at page 19, lines 1-10, and, in its place, add the following new paragraph:

- 9 -

A further aspect of the invention provides a method for assessing the activity of a first protein kinase and a second protein kinase, comprising the steps of exposing the first protein kinase to a first polypeptide of a kit of the first aspect of the invention, and exposing the second protein kinase to a second polypeptide of a kit of the first aspect of the invention; and determining whether and optionally to what extent the said polypeptide is phosphorylated. In a particularly preferred embodiment, the polypeptide of the kit are polypeptides of the invention, as discussed above, ie comprise the amino acid sequence LSFAEPG (SEQ ID NO:6).

Please cancel the paragraph at page 19, lines 19-27, and, in its place, add the following new paragraph:

Thus, a further aspect of the invention provides a method for characterising the substrate specificity of a protein kinase, comprising the steps of exposing the protein kinase to a first polypeptide of a kit of the first aspect of the invention, and exposing the protein kinase to a second polypeptide of a kit of the first aspect of the invention; and determining whether and optionally to what extent the said polypeptides are phosphorylated. In a particularly preferred

- 10 -

embodiment, the specified polypeptides of the kit are unphosphorylated polypeptides of the invention, ie comprising the amino acid sequence LSFAEPG (SEQ ID NO:6).

Please cancel the paragraph at page 21, lines 22-30, and, in its place, add the following new paragraph:

Preferred protein kinases for assay by the methods of the invention (particularly when using a polypeptide comprising the epitope LSFAEPG (SEQ ID NO:6)) include protein kinases that do not have a strong sequence preference C-terminal of the phosphorylated residue, for example members of the PKA family (including, for example, isoforms of PKG, ROCKII and PKC), PKB family (including, for example, isoforms of PKB, SGK, MSK, MAPKAP-K1 and S6K) or MAPKAP-K2 family (including, for example, MAPKAP-K3, PRAK, CHK1, CHK2, AMPK and CaMKII) or CK1 isoforms and subfamily.

Please cancel the paragraph at page 25, lines 4-11, and, in its place, add the following new paragraph:

- 11 -

Fig 1. Generation of a phospho-specific antibody that recognises the epitope LpSFAEPG (SEQ ID NO:7).

Aliquots (10 ng or 100 ng) of the peptide LpSFAEPGC (SEQ ID NO:17) conjugated to bovine serum albumin were spotted on to a nitrocellulose membrane and immunoblotted with the phospho-specific antibody raised against the phospho-peptide immunogen in the presence or absence of the peptides shown on the right. The prefix "p" before S denotes the serine-phosphorylated form of the peptide.

Please cancel the paragraph at page 25, lines 13-22, and, in its place, add the following new paragraph:

Fig 2. Comparison of the new ELISA based protein kinase-based assay with the standard radioactive filter-binding assay.

The protein kinases indicated were assayed by the ELISA-method (open bars) or standard radioactive assay (filled bars) using the peptide substrates RARTLSFAEPG (SEQ ID NO:16) (A), KKLNRTLSFAEPG (SEQ ID NO:13) (B) and RRRLSFAEPG (SEQ ID NO:4) (C) and in the presence or absence of the kinase inhibitors shown. The results are shown relative to control incubations in the absence of inhibitors and are

- 12 -

presented as standard error of the mean for four determinations (two independent experiments). Similar results were obtained in several further experiments.

Please cancel the paragraph at page 28, lines 12-25, and, in its place, add the following new paragraph:

Antibodies

Antibodies that recognise the common seven residue phosphorylated motif (LpSFAEPG) (SEQ ID NO:7) contained within each of the three generic peptide substrates described under results, were raised against the peptide LpSFAEPGC (SEQ ID NO:17) (where pS represents phosphoserine). The C-terminal cysteine residue was added to enable coupling to keyhole limpet haemocyanin. The peptide-protein conjugate was injected into sheep at Diagnostic Scotland (Carluke, U.K.) and the anti-sera purified on protein G-Sepharose (Amersham Pharmacia Biotech) by Dr J. Leitch in this Unit. Antibodies were used at a concentration of 2 μ g/ml for immunoblotting and 10-20 μ g/ml for detection of phosphorylated peptides in the modified ELISA format. Rabbit anti-sheep antibodies conjugated to peroxidase were obtained from Pierce (Tattenhall, Cheshire, UK) and used at 1/10,000 dilution.

- 13 -

Please cancel the paragraph at page 30, line 14 to page 31, line 2, and, in its place, add the following new paragraph:

PKB α , SGK1, MSK1, S6K1, PKA, PKG, ROCKII, MAPKAP-K2, MAPKAP-K3, PRAK, CHK1 and CHK2 were assayed in 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1 % (v/v) β -mercaptoethanol, 0.01 % (w/v) Brij-35, the AMPK in 50 mM Hepes pH 7.4, 1 mM dithiothreitol, 0.02% Brij-35 and 0.2 mM AMP, PKC α in 20 mM Hepes, pH 7.4, 0.03% Triton X-100, 0.1 mM CaCl₂, 0.1 mg/ml phosphatidylserine and 10 μ g/ml 1,2-dioleoyl-*sn*-glycerol and CaMKII in 50 mM Hepes, pH 7.4, 5 mM CaCl₂, 0.03 mg/ml calmodulin. The V_{max} values for the three generic peptides developed in this example are given relative to the standard peptide substrates for each protein kinase. The standard peptide substrates (single amino acid code) were:- GRPRTSSFAEG (SEQ ID NO:18) (PKB α , SGK1), LRRASLG (SEQ ID NO:19) (MSK1, S6K1, PKA), KEAKEKRQEQIAKRRRLSSLRASTSKSGGSQK (SEQ ID NO:20) (ROCK-II and PKG), KKLNRRTLSVA (SEQ ID NO:21) (MAPKAP-K2 and MAPKAP-K3), KKLRRRTLSVA (SEQ ID NO:22) (PRAK); KKKVSRSGLYRSPSPENLNRPR (SEQ ID NO:23) (CHK1 and CHK2), HMRSAMSGLHLVKRR (SEQ ID NO:24) (AMPK), MHRQETVDCLK (SEQ ID NO:25) (CaM-KII), histone H1 (PKC).

Please cancel Table 1 on pages 31-32, and, in its place, add the following new Table 1:

Table 1

A.

Enzyme	RARTLSFAEPG (SEQ ID NO:16)		Standard Substrate	
	Km (μ M)	Relative Vmax (%)	Km (μ M)	Relative Vmax (%)
MAPKAP-K2	5	52	7	100
MAPKAP-K3	30	105	18	100
PRAK	40	40	>100	100
CHK1	5	117	5	100
CHK2	>300	221	>300	100
AMPK	75	114	80	100
CaMKII	300	80	60	100

B.

Enzyme	KKLNRTLFAEPG (SEQ ID NO:13)	Standard Substrate
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	Km (μ M)	Relative Vmax (%)	Km (μ M)	Relative Vmax (%)
PKB α	2	100	4	100
SGK1	4	105	4	100
MSK1	2	156	6	100
S6K1	6	124	8	100

C.

Enzyme	RRRLSFAEPG (SEQ ID No 4)		Standard Substrate	
	Km (μ M)	Relative Vmax (%)	Km (μ M)	Relative Vmax (%)
PKA	30	161	18	100
PKG	50	122	5	100
ROCKII	>100	39	5	100
PKC α	>500	39	2	100

Please cancel the paragraph at page 30 lines 6-11, and, in its place, add the following new paragraph:

Although PKC could be assayed perfectly well using the peptide Arg-Arg-Arg-Leu-Ser-Phe-Ala-Glu-Pro Gly (SEQ ID NO:4) (Table 1), this substrate was inferior to other substrates that have been used to assay this protein kinase. This is because PKC prefers basic residues C-terminal to the site of phosphorylation, which

- 16 -

are not present in the epitope, in addition basic residues N-terminal to the site of phosphorylation [5].

Please cancel the paragraph at page 32, line 13 to page 33, line 12, and, in its place, add the following new paragraph:

Generation of a phospho-specific antibody to the common C-terminal epitope.

The major purpose of the present study was to simplify "high throughput screening" of protein kinases by developing a non-radioactive assay applicable to the assay of many protein kinases. In order to do this, we therefore generated a phospho-specific antibody capable of recognising the phosphorylated epitope (LpSFAEPG) (SEQ ID NO:7) common to the three generic substrates (see Methods). This antibody recognised <10 ng of the conjugated phosphopeptide antigen (Fig 1). The ability of the antibody to recognise the three generic substrate peptides in their phosphorylated form was established by competition studies. These experiments showed that the phosphopeptide antibody was not only neutralised by pre-incubation with the seven residue phospho-peptide antigen, but also by each of the phosphorylated generic peptide

- 17 -

substrates. In contrast, the unphosphorylated forms of the same peptides were unable to neutralise the antibody (Fig 1).

Please cancel the paragraph at page 33, line 25 to page 34, line 20, and, in its place, add the following new paragraph:

Concluding remarks.

In this example we have described a simple non-radioactive assay that is suitable for the high throughput screening of many protein kinases. We consider that the same three peptides can be used as substrates by all the closely related isoforms the protein kinases studied, and probably by most, if not all, of the other members of the same kinase subfamilies. We also consider that additional peptides with the same C-terminal epitope may be used to assay other protein kinases that recognise a specific motif N-terminal to the site of phosphorylation. For example, CK1 (previous called casein kinase1) phosphorylates serine residues that lie three residues C-terminal to another phosphoserine residue [10]. It may therefore phosphorylate peptides of the type Xaa-pSer-Xaa-Leu-Ser-Phe-Ala-Glu-Pro-Gly (SEQ ID NO:12). We therefore consider

that our strategy is applicable to at least 50 and probably more than 100 protein kinases. The assay we have developed is not applicable to protein kinases that have a stringent requirement for a particular motif or residue C-terminal to the site of phosphorylation. For example, MAP kinases and cyclin-dependent protein kinases have an absolute requirement for a proline residue immediately C-terminal to the site of phosphorylation [5], which is a negative determinant for the protein kinases tested in this example. Similarly CK2 requires several consecutive acidic residues C-terminal to the site of phosphorylation, while the DNA-dependent protein kinase requires a C-terminal glutamine [5]. However, in view of the results presented herein, a second series of peptide substrates with a common N-terminal epitope may be useful in assaying many protein kinases that require a specific C-terminal motif for phosphorylation.